33PTO 07-5738

CC=JP DATE=19741111 KIND=A
PN=49117688

METHOD FOR MANUFACTURING DIFRUCTOSE DIANHYDRIDE III
[Jifurukuto-su·Jianhidorido III No Siezoho]

Kuniji Tanaka

UNITED STATES PATENT AND TRADEMARK OFFICE
Washington, D.C. July 2007

Translated by: FLS, Inc.

PUBLICATION COUNTRY	(19):	JP
DOCUMENT NUMBER	(11):	49117688
DOCUMENT KIND	(12):	A
PUBLICATION DATE	(43):	19741111
PUBLICATION DATE	(45):	
APPLICATION NUMBER	(21):	48031086
APPLICATION DATE	(22):	19730317
INTERNATIONAL CLASSIFICATION	(51):	
DOMESTIC CLASSIFICATION	(52):	36(2)D522; 16E44; 34K2
INVENTOR	(72):	TANAKA; KUNIJI
APPLICANT	(71):	KAKEN PHARMACEUTICAL CO., LTD.
TITLE	(54):	METHOD FOR MANUFACTURING DIFRUCTOSE DIANHYDRIDE III
FOREIGN TITLE	[54A]:	JIFURUKUTO-SU·JIANHIDORIDO III NO SEIZOHO

Specifications /517

1. Title of Invention

Method for Manufacturing Difructose Dianhyride

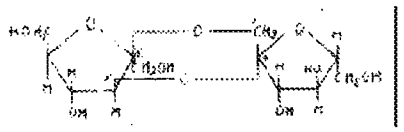
2. Claim(s)

A method for manufacturing difructose dianhyride III characterized by using inulin or an inulin-containing plant extract as the raw material and utilizing cells belonging to Aurthobacter ureafaciens or an enzyme producing them.

3. Detailed Specifications

The present invention relates to a method for manufacturing difructose dianhyride III (referred to as "DFA III" hereinafter) at a high yield from inulin or an inulin-containing plant extract by utilizing a microorganism or an enzyme producing it.

DFA III is a disaccharide having the structure shown by the following formula:



Di-D-fructofuranose-1,2':2,3'-dianhydride

It has been isolated and identified by Jackson, et al. (see Bur. Stand. J. Res. 6 (1931):709). This substance has considerable sweetness and at the same time is safer than other saccharides, as is assumed from the aforesaid structure, and is not metabolized by animals. Consequently, it has been investigated as a non-calorie sweetener and is useful in applications for diabetes, for foods for cosmetics, etc.

---

[1]Number in the margin indicates pagination in the foreign text.

Inulin is a polysaccharide using only fructose as the constitutive sugar. Although Jackson, et al., have isolated DFA III from a hydrolysate of inulin using an acid, the yield thereof is less than 2% and hardly any of it is fructose. As a consequence, a method for removing inulin with acid of is not suitable at least as a method for manufacturing DFA III.

As a result of repeating various research to solve these problems, the inventors of the present invention discovered that DFA III could /518 be obtained at a high yield from inulin or an inulin-containing plant extract by using a certain kind of microorganism.

This kind of microorganism was isolated from soil in Akashi City, Kokubo, Hyogo Prefecture. An enzyme having action for directly decomposing inulin as far as fructose, such as inulase, normally produces a different type of inulin catabolic enzyme. Thus, DFA III can be obtained by digesting inulin with this bacterium an inulin catabolic enzyme produced by this bacterium. This type of sugar (called "Species No. 7116" tentatively) also is deposited at the Fermentation Research Institute, the Agency of Industrial Science and Technology based on FERM No. 1969. The mycological properties of Species No. 7116 will now be listed.

(1) Morphological properties

Morphology: 0.1 to 0.2 by 1.0 to 1.5 μ bacillus; spore: not produced; flagellum: none; Gram stain: weakly acidic; acid-resistant: none

(2) Cultural properties

Stab culture in broth gelatin: liquefied; agar [illegible]: circular form, hilly, smooth, completely round, glossy, buttery, opaque, dull yellow;

agar gradient: moderate growth, filiform, glossy, buttery, dull yellow; broth: moderate growth; potato: moderate growth; litmus milk: slight change; BCP milk

(3) Physiological properties:

Aerobic; exhibits satisfactory growth at 30°C; production of indole: present (in cysteine-added broth); hydrocarbon production: acid and gas not produced from any of the below-mentioned hydrocarbons: arabinose, xylose, glucose, mannose, lactose, galactose, lactose, maltose, saccharose, trehalose, raffinose, sorbitol, inositol, glycerol, salicin, α-methylglucoside, inulin, dextrin, starch, cellulose; production of acetylmethyl carbinol: none; degradation of starch: none; reducibility of nitrate: present; production of ammonia: none; Methyl Red reaction: negative; utilization for citrate: present (on Christenssen medium); utilization for ammonium salts: present (on [illegible] medium); Methylene Blue reducibility: none; 2,6-dichlorophenolindophenol reducibility: present; degradation of casein: present; production of catalase: present

In accordance with the various attributes above, since this bacteria is a weak Gram-positive bacillus according to a search of Bergey's Manual of Determinative Bacteriology, 7th edition (1975), there is no production of [illegible] from [illegible], it [illegible] gelatin and grows on a [illegible] medium, and so forth, it is thought to belong to the genus Arthrobacter. Furthermore, in a search of well-known species as described in Bergey's Manual Systematic Bacteriology, it is chromogenic, does not decompose starches, does not reduce nitrates, and exhibits a yellow color; hence, it is assumed to be Arthrobacter ureafaciens. Upon comparison,

these mycological properties agree extremely well with the mycological properties of Species No. 7116 above and there are no differences in the described items.

Consequently, Species No. 7116 was assumed to be Arthrobacter ureafaciens (Krebes and Eggleston (1939) and Clark (1955)).

Although it is commercially-available [illegible] in an inulin-containing root or rhizome of a plant belonging to the Asteraceae family, such as Jerusalem Artichoke or burdock root, [illegible], this strain is [illegible] in an aqueous inulin solution. A culturing temperature of 37°C is favorable. The production of DFA III can be traced according to its optical rotation, for the reason that an aqueous inulin /519 solution is levorotary or DFA III is weakly dextrorotary. Therefore, where the optical rogation is weakly dextrorotary, and thus fixed, digestion may be suspended by heating. The time needed for such culturing is normally 5 to 10 days. Of course, measurement of the optical rotation during digestion is not required as long as it is completely standardized.

After removing the biomass by filtration upon adding a filtration accelerator, such as High-Flow Super Cell (made by Wako Pure Chemical Industries, Ltd.), a bread yeast is added, and removing the fructose besides that of DFA III and other oligosaccharides by fermentation is effective for increasing the DFA III purity. After the fermentation ends, the bread yeast also is filtered upon adding a filtration accelerator. Moreover, removal of the microorganism in these processing steps naturally can be replaced by a centrifuging operation. This filtrate is concentrated, adsorbed onto an active carbon column by active carbon column chromatography,

and DFA III can be obtained in the fraction eluted in a 5% aqueous ethanol solution. The anticipated DFA III is obtained by concentrating and hardening this. In addition, an inulin-degrading enzyme (referred to as "crude enzyme" hereinafter) produced by this bacteria species by adding ammonium sulfate (65% saturated) to the digested culture, filtering the deposited precipitate several times, dialyzing the filtrate thereof, and subsequently freeze-drying it can be obtained. The optimum pH of this crude enzyme is 6.5 to 7.5 for it to act specifically on inulin. Consequently, the sought-after DFA III may be produced by acting a commercially-available inulin in a buffer whose pH has been adjusted to 7.0 on the crude enzyme.

It is sweet (about half as sweet as sucrose), it has no reducing power as is, and fructose is produced by hydrolysis with an acid. And it dissolves well in water.

Optical rotation $[\alpha]_D^{20}$ = 136°; melting point: 162°C

The Hf according to thin-layer chromatography is 0.63 to 0.64 at conditions including an Avicel SF (sold by Hunakoshi Ltd.) and n-butanol:pyridine:water (=6:4:3), which agreed well with that of a standard DFA III substance.

Next, the method of the present invention will be described specifically by citing practical examples.

Practical Example 1

After washing commercially-available burdock root and chopping 200 g of it finely, 500 mL of distilled water are added thereto, and subjected to an extraction with boiling water for 1 hour. After cooling, it is filtered with gauze to obtain a filtrate. The pH of this filtrate is

adjusted to 7.0 with 1[illegible] NaOH, subsequently transferred to a sterile flask, and this is subjected to a high-pressure sterilization for 20 minutes at conditions of 120°C and 2 atmospheres. This sterilized extract is inoculated with Species No. 7116 using several platinum loops, and allowed to stand still at 37°C to culture it. The culture liquid is removed during the culturing using a sterilized injector, the removed liquid is centrifuged, the supernatant thereof is heat treated, after which the optical rotation is measured to comprehend the amount of DFA III produced. The dextrorotation increases easily and is fixed. At this time, digestion is stopped. 1.5 g of High-Flow Super Cell are added to the culture liquid after 6 days, then filtered to remove the biomass. This filtrate is aged for 10 minutes to inactivate the enzyme, after which about 20 g of a bread yeast were added to this, allowed to stand still for 2 hours, after which High-Flow Super Cell is added and then filtered. This filtrate is concentrated to 70 mL under reduced pressure. This liquid is adsorbed onto an active carbon column (column diameter: 2.5 cm, column height: 45 cm; packed with a mixture of 30 g active carbon and 60 g of No. 535 Celite with distilled water), 1.3 L of distilled water was poured thereinto, and subsequently eluted with a [illegible]% aqueous ethanol solution. Roughly 15 mL at a time of the eluate is collected to measure the amount of DFA III based on the optical rotation. The elution peaks (fraction nos. 15 to 50) thereof are collected and dried by concentration under reduced pressure. (yield: 0.5 g; $[\alpha]_D^{30}$: 126.5°)

Elemental analysis values:

Theoretical value (%): C 44.44 H 6.17

Actual values (%): C 44.15 H 5.20

Melting point: 154 to 155°C

Practical Example 2

After drying Jerusalem Artichoke, 150 g thereof is subsequently chopped finely, then boiled and extracted for 1 hour with 650 mL distilled water. After cooling, it is filtered with gauze to obtain a filtrate. The pH of this filtrate is adjusted to 6.0 with 1N NaOH, subsequently transferred to a sterile flask, and this is subjected to a high-pressure sterilization for 20 minutes at conditions of 120°C and 2 atmospheres. This /52C sterilized extract is inoculated with Species No. 7116 using several platinum loops, and allowed to stand still at 37°C to culture it. The timely optical rotation is measured as in Practical Example 1. 1.5 g of High-Flow Super Cell are added to this culture liquid after 9 hours and then filtered to remove the biomass. 100 mL of this filtrate are removed and heated for 10 minutes to inactivate the enzyme, about 15 g of a bread yeast are added thereto, and allowed to stand still for 2 hours, High-Flow Super Cell is subsequently added and then filtered. 50 mL of this filtrate are concentrated to about 10 mL under reduced pressure. This liquid is adsorbed onto an inactive carbon column (same as that in Practical Example 1), 1.3 L of distilled water is poured thereinto, and subsequently eluted with a 5% aqueous ethanol solution. 15 mL of the eluate is collected at a time to measure the amount of DFA III based on the optical rotation. The elution peaks (fraction nos. 10 to 50) are collected and dried solid by concentration under a reduced pressure. (yield: 0.5 g, $[\alpha]_D^{20}$: 127.3°)

Theoretical value (%): C 44.44 H 6.17

Actual values (%): C 44.15 H 6.10

Melting point: 155 to 156°C

Practical Example 3

300 mL of the digested culture liquid in Practical Example 2 are removed, 65 g ammonium sulfate (65% saturated) are added under stirring, then set aside in a refrigerator overnight. 5 g of High-Flow Super Cell are added to the deposited precipitate and stirred and subsequently filtered. This filtered residue is washed with 20 mL of water and subjected to shaking for 15 minutes. This is filtered, and moreover, the residue is washed with a small amount of water, after which it is dialyzed in a refrigerator for 24 hours with distilled water and subsequently freeze-dried. 30 mg of a crude enzyme are obtained therefrom.

2 g of a commercially-available inulin are dissolved in 100 mL of a 0.1 mol acetate buffer, the previous crude enzyme is added, the temperature is raised once to 65°C for 10 minutes to inactivate the so-called inulase, a small amount of toluene is added to the supernatant, and this allowed to stand still for 6 days at 30°C. After that, 10 g of a bread enzyme are added and the temperature is maintained at 37°C for 2 hours. Then 1 g of High-Flow Super Cell is added and then filtered, after which the filtrate is concentrated under reduced pressure. This is subjected to the same inactive carbon column as in the previous examples to obtain 0.5 g DFA III. ($[\alpha]_D^{20} = 126.2°$)

Elemental analysis values:

Theoretical values (%): C 44.44 H 6.17

Actual values (%): C 44.15 H 6.10

Melting point: 158 °C

Practical Example 4

The pH is adjusted to 7.0 with 2N NaOH in a solution in which 15 g inulin, 2 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.5 g KH_2PO_4 and several mg of $FeSO_4$ are dissolved in 1L of distilled water, after which it is concentrated at high pressure for 20 minutes at conditions of 120°C and 2 atmospheres. This sterilized solution is inoculated with Species No. 7116 using several platinum loops, then set aside at 37°C to culture it. The timely optical rotation is measured during culturing, as in the practical examples, to measure the production of DFA III. 2 g of High-Flow Super Cell are added to the culture liquid after 5 days, then filtered to remove the biomass. This is heated for 10 minutes to inactivate the enzyme after which 20 g of a bread yeast are added thereto and allowed to stand still for 2 hours. The enzyme is subsequently removed by centrifugation, 200 mL of the supernatant is removed and concentrated to about 10 mL under reduced pressure, then adsorbed onto an inactive charcoal column as in Practical Example 1. 1.3 L of distilled water is poured in and eluted with a 5% aqueous ethanol solution. 15 mL at a time of the eluate is collected to measure the amount of DFA III based on the optical rotation. The elution peaks thereof (fraction nos. 10 to 55) are collected, then concentrated under reduced pressure and dried solid. (yield: 0.7 g, $[\alpha]_D^{20} = 127.0°$)

Elemental analysis values:

Theoretical values(%): C 44.44 H 6.17

Actual values(%): C 44.35 H 6.15

Melting point: 158 °C